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(54) **HUMAN SOURCE LEADING SEQUENCE, GENE VECTOR AND GENE EXPRESSION STRATEGY**

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(52) **U.S. Cl.** **435/6**; 435/91.1; 435/91.2

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(57) **ABSTRACT**

The present invention provides a human source gene targeting sequence a gene vector and gene expression strategies. The invention includes the following: (1) Using a DNA sequence without important physiological function-related genes in the short arms of human group D, G chromosomes, or a DNA sequence sharing 50% or over 50% identity to the sequence selected from human D, G group chromosomes, as a targeting sequence for gene targeting and (2) Construction of a gene vector containing the targeting sequence. the nucleolus organizing region in D, G group chromosomes that is described above is used as the target site, the gene of interest is integrated into the short arms where the gene expresses actively in D,G group chromosomes of human cells. The present invention provides a novel gene targeting sequence by which the gene vector construction and gene expression strategies are realized. The gene expression strategies can be used for human gene therapy and for manufacturing protein.

2 Claims, 2 Drawing Sheets

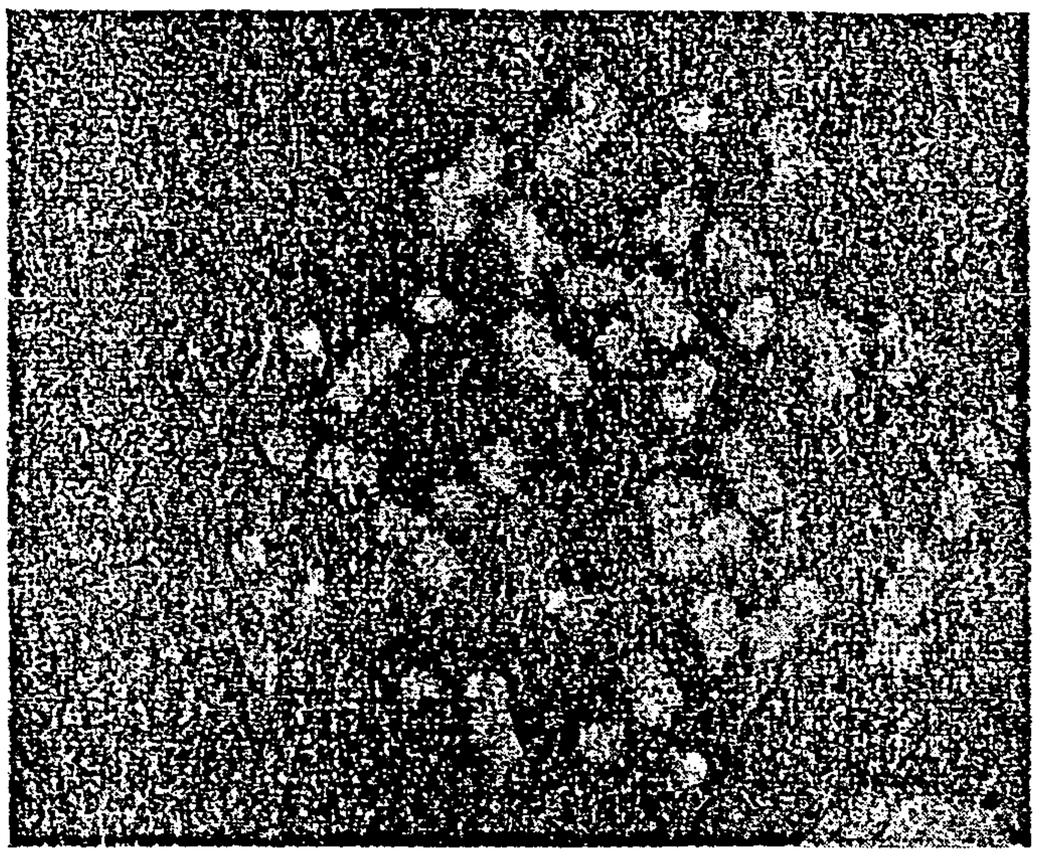

Fig. 1

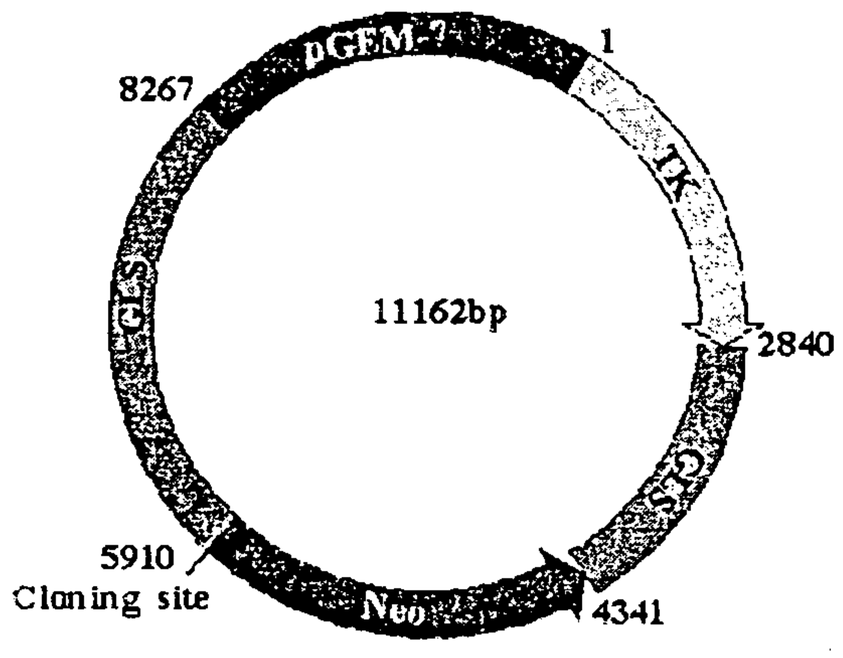

Fig.2

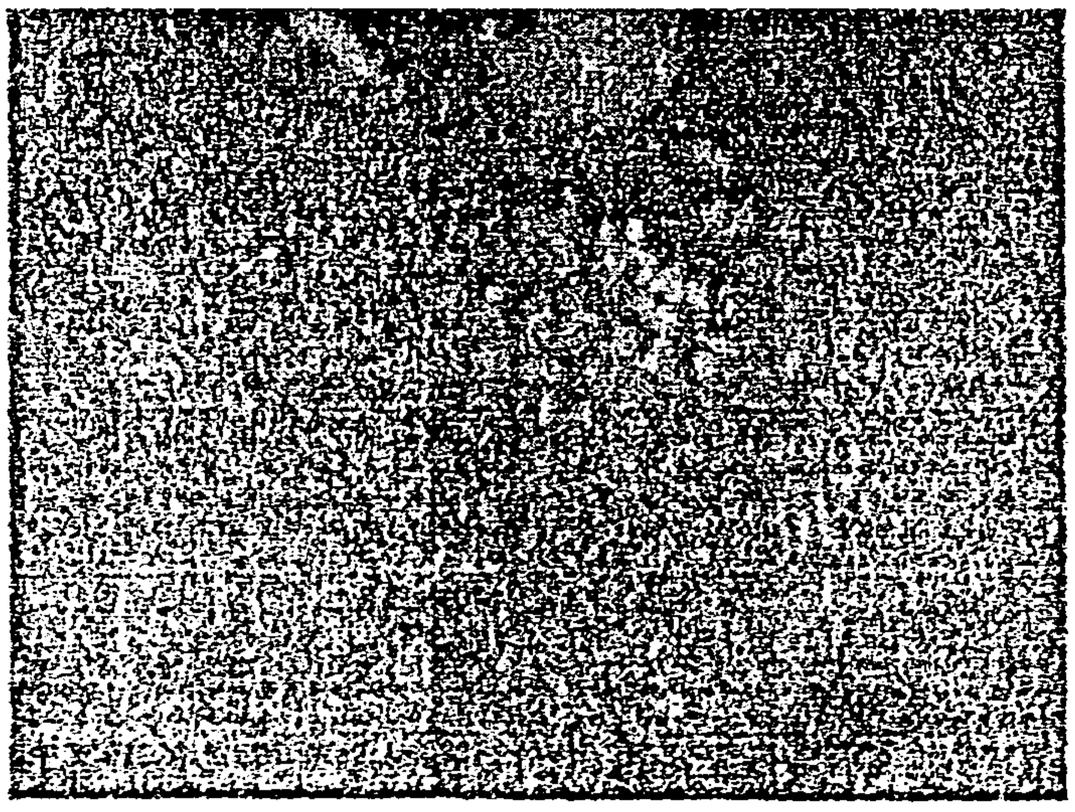

Fig. 3

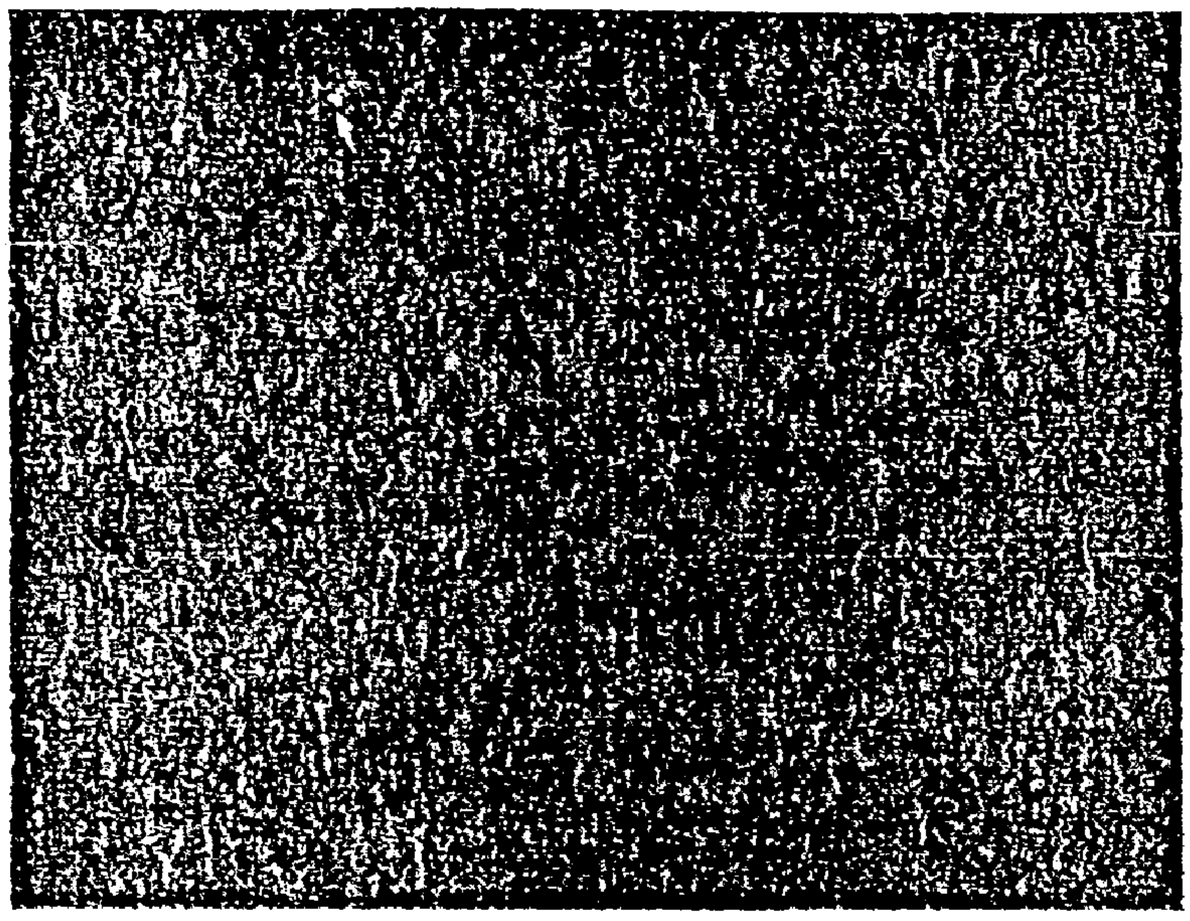

Fig.4

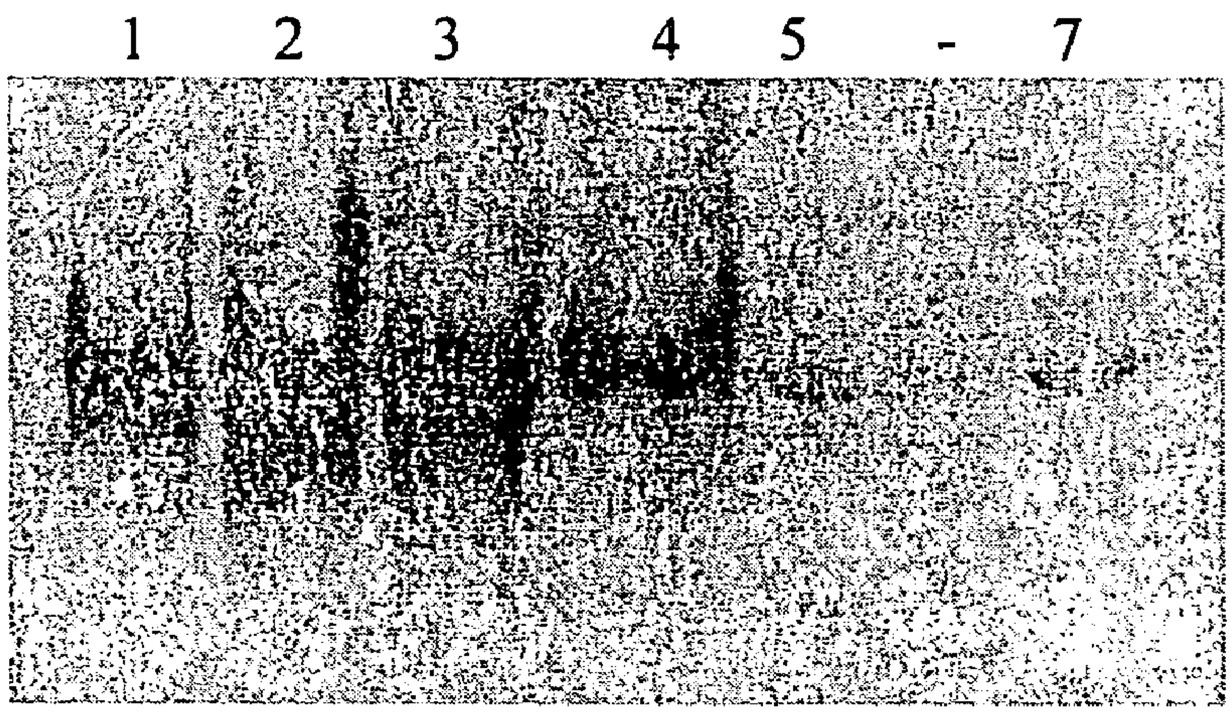

Fig.5

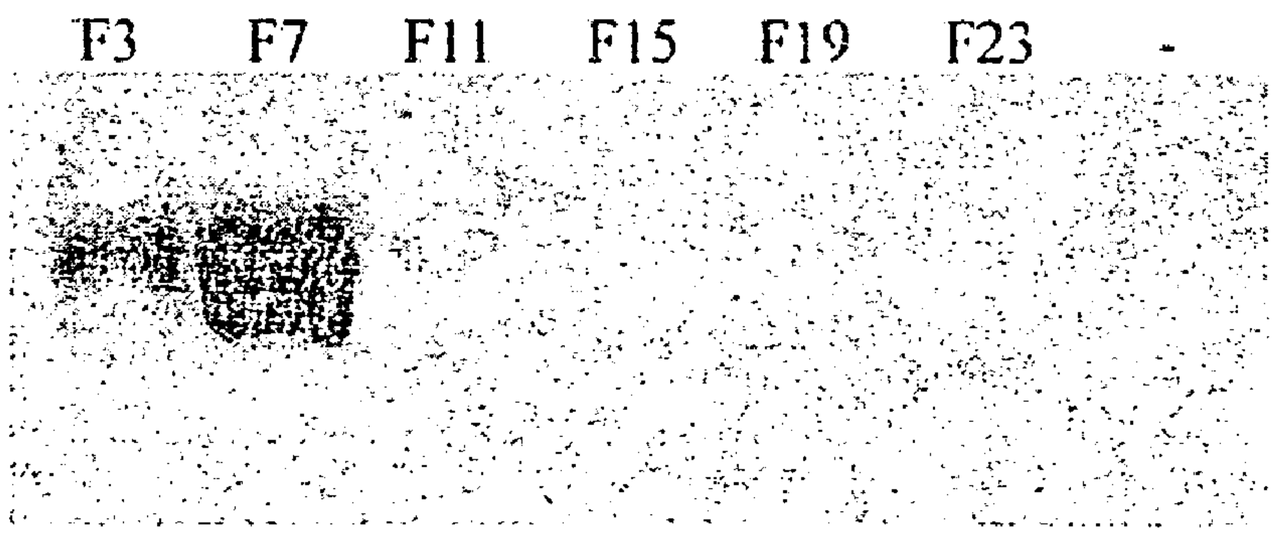

Fig.6

HUMAN SOURCE LEADING SEQUENCE, GENE VECTOR AND GENE EXPRESSION STRATEGY

RELATED APPLICATIONS

This application is the national phase under 35 U.S.C. § 371 of PCT International Application No. PCT/CN01/00126 which has an International filing date of Feb. 16, 2001, which designated the United States of America.

SUMMARY OF THE INVENTION

The invention deals with gene targetinger sequence, by which a gene vector was constructed. The invention also relates to a strategy for expression of a target gene introduced by the gene vector.

BACKGROUND OF THE INVENTION

Statistical data of Mendelian inheritance in man demonstrates, up to now, that 1660 single gene disorders have been identified and 989 disease-related genes have been identified before Jun. 30, 2000. Most recessive genetic disorders may be treated by introducing the normal gene into cells of the patient. With the advancement of research, gene therapy regarding dominant genetic disorders and somatic cell-related tumors has begun. In 1995, E. Marshall put forward in *Science* that the key point of gene therapy research was novel vector development and discovery, but the problem of an effective gene therapy vector remain unsolved so far. The main reason is that the researchers do not step out of the circle of constructing a vector using viral components. General speaking, commonly-used viral vectors have many defects as follows: 1) Instability: gene insertion efficiency is low, and the vector exists in the cell nucleus partially as a form of an attached body that is not inherited stably during cell division. As a result, the therapeutic gene cannot be expressed for a long time and in a stable manner. 2) Poor safety: for example, mutation caused by random insertion may affect function of normal genes at integration sites, and even activate an oncogene, which may result in other diseases and tumors respectively. Furthermore, viral vectors may generate wild-type recombinant virus with replication ability and so harm the patient. In recent years, it was reported that an adenovirus vector caused a patient death during the conduct of a gene therapy. 3) Immunogenicity: the proteins produced by viral genes and protein contaminating the vector during purification could induce an immunogenic reaction and influence the expression of the therapeutic gene.

In the middle of the 1980's, a gene targeting vector was developed based on the principle of homologous recombination to achieve site-directed integration, which could avoid immunogenicity and random integration, but the gene targeting vector used for site-directed repair in gene therapy and replacement of a defective gene have to utilize specific fragments of the two sides of the gene as targeting sequences, therefore its application is limited and the transfection efficiency is still low. In fact, it is useful for gene knock-out in embryonic stem cells and fertilized egg cells, but is not suitable for site-directed integration in mature somatic cells (Galli-Taliadoros L A, Sedgwick J W, Wood S A, et al. J Immunol Meth 1995, 181:1-15; Hasty P, Rivera-Perez J, Chang C, et al. Mol Cell Biol 1991, 11(9):4509-4517).

Rosenbery concluded that no case had shown certain clinical efficacy among hundreds of gene therapy experiments (Rosenbery L E & Schechter A. N. Science, 2000; 287:1751). Thus, development of a novel, stably inherited gene vector causing no harm to a human body remains a key problem to be solved.

DETAILED DESCRIPTION OF THE INVENTION

In 1981, the applicant found two families carrying a rarely reported bi-satellite microchromosome (BM), but the phenotype is normal. The microchromosomes have been stably inherited in two families over 2 and 3 generations and show no harm to a human body. Through document investigation, we found that there were seven similar families in both Europe and USA. Up to now, total 17 families have been reported, but no one has thought to use this chromosome as a source of components for gene vector construction. The inventor put forward in 1991 a proposal for dissection of components this chromosome and construction of a gene vector having a human source. The project was initiated in 1994 and executed in 1995. The applicant first detected during investigation that the bi-satellite microchromosomes (BM) originated from the short arms of human D and G group chromosomes, including chromosomes 13, 14, 15, 21 and 22. The short arms of D, G group chromosomes contain a nucleolus organizing region and are rich in ribosomal DNA. Preliminary biological research has revealed that polymorphism of different lengths (namely containing different concentrations of rDNA) were commonly found in this region in the population, and the genes here could be transcribed very actively during cell division. Therefore, the applicant inferred that if a specific fragment from the BM could be isolated and used as a targeting sequence, a foreign gene can then be transferred in a site-directed manner into the nucleolus organizing region, and the expression of the gene should be effective, stable and unharmful. Subsequent experiments have strongly proved that inference.

It is of great significance to find the BM and isolate specific DNA fragments showing homology to the nucleolus organizing region in the short arms of human D, G group chromosomes.

The applicant first constructed a BM-specific pUC19 library by micro-dissection, PCR and microcloning techniques, and then isolated a single copy fragment by screening this library. The single copy fragment was proved to be from BM and the short arms of D, G group chromosomes by Fluorescent In Situ Hybridization (FISH). The single copy fragment was further used as probe to screen a PAC genome library and thus a DNA fragment of 120 kb (BMSF) (SEQ No.1) was obtained. This DNA fragment was also confirmed to originate from the short arms of human D, G group chromosomes (FIG. **1**). Sequence analysis indicated no physiological function-related genes were found in the BMSF fragment, suggesting the target site is safe.

It is within the aim of the invention to isolate a DNA sequence that does not contain an important physiological function-related gene from the short arms of human D, G group chromosomes or a sequence sharing 50% or great than 50% similarity to short arms in human D, G group chromosomes as a gene targeting sequence. For example, in example 1, a sequence was selected from SEQ ID NO: 1 to construct a gene vector having a human source. This sequence comes from the short arms of human D, G group chromosomes.

The 120 kb DNA fragment above can be used as gene targeting sequence, and a smaller fragment with specificity can also be selected from the 120 kb DNA fragment. In the applied case, a 3.8 kb fragment from nucleotide 75590 to 79448 in the SEQ No. 1 is selected as a gene targeting sequence (GLS). According to the requirements of gene vector construction, positive and negative screening genes should be added to the vector, too.

More concretely, to construct a human gene therapy vector, the 3.8 kb fragment from nucleotide 75590 to 79448 of SEQ ID NO: 1 was inserted into the pGEM-TK vector, which contained a negative screening thymidine kinase gene (TK). A positive screening gene Neo was inserted into site 1500 of the GLS which divided the GLS into two arms of 1.5 kb and 2.3 Kb. Thus, the gene vector was constructed. The bacterial strain containing the gene vector was deposited in the China Typical Culture Collection Center on Sep. 29, 2000 (Wuhan University, Wuhan 430072, China). The accession number is CCTCCM200030. The administrator designated the reserve classification nomenclature which is *Escherichia coli* JM109/JH-4/pNS2. FIG. **2** shows the vector, and the sequence is given in SEQ ID NO: 2.

This present invention provides a specific target site coming from SEQ ID NO: 1 which denotes a DNA targeting sequence originated from the short arms of a D, G group chromosome. The gene vector comprising the fragment above can transfer a gene of interest into specific target sites which are include no important physiological function-related genes, therefore targeting is safe.

Based on the invention technology, the applicant further put forward the following procedures for gene expression.

(1) A gene vector is constructed using a DNA sequence with no vital physiological function-related genes obtained from the short arms of Group D,G chromosomes of a human being, or by using a targeting sequence which is 50% or greater than 50% homologous to such a DNA sequence;

(2) Clone a gene of interest into the above-described gene vector;

(3) Transfer the gene of interest into target sites of the nucleolus organizing region of group D,G chromosomes of host cells;

(4) Express the gene of interest in vivo and in vitro.

All procedures including gene vector construction, recombination of gene of interest into the vector, transfer of gene of interest into host cells and expression of the gene of interest can be conducted by conventional technologies.

The targeting sequence of the gene of interest can be a portion of the sequence selected from the DNA sequence of SEQ ID NO: 1. Preferably, the targeting sequence of the gene of interest is the portion at the positions from 75590 to 79448 SEQ ID NO: 1.

The gene targeting sequence above can be used to construct a gene vector containing positive and negative screening genes.

The examples 2 and 3 of the present invention explain the procedures above:

① A preferred specific DNA fragment of 3.8 kb selected from BMSF of 120 Kb is subcloned into a pGEM-TK plasmid vector, which uses TK as a negative screening gene. A positive screening gene Neo was inserted into the 3.8 kb DNA fragment to construct a gene vector. ② A gene of interest digested by a restriction enzyme is ligated into the exogenous gene cloning site on one side of the Neo gene. The existence of all parts and their orientation is confirmed by polymerase chain reaction. ③ A single restriction enzyme digestion site in the pGEM vector selected and the gene vector is linearized. The gene vector is transferred into target cells by means of electroporation or liposome transfection. ④ Transformed target cells are screened using G418 and GCV to obtain site-directed integrating positive cells clones. ⑤ The expression of the gene of interest is detected from positive cell clones so as to obtain site-directed integration into the short arms of D, G group chromosomes and stable expression of the gene of interest.

The positive cells expressing the gene of interest obtained by the screening are embedded hypodermically or injected intravenously into the body or the gene of interest encapsulated by liposomes is directly injected into the body so that gene is expressed for a long time and stably within the body to correct the clinical symptom caused by the defective gene.

The example of the present invention provides a DNA sequence of a gene vector as shown as SEQ ID NO: 2, of which the targeting sequence is from 75590 to 79448 of SEQ ID NO: 1. TK is a negative screening gene. The positive screening gene Neo is inserted into site 1500 of the GLS and the GLS is divided into two arms of 1.5 kb and 2.3 kb. The cloning site is at nucleotide 5910. The example of the present invention made public in vitro expression in HT1080 cells of genes of tissue-type plasminogen activator (TPA) used for treatment of lood occlusion disorders and coagulant factor IX (FIX) used for treatment of haemophilia B. These experiments showed the expression is both efficient and stable.

Therefore, gene expression strategies provided by the present invention are of great promise in practical applications. The gene expression strategies are not only used for manufacturing medicinal protein but also give an effective way for gene therapy.

The inventor made use of a chromosome segment not harmful to the human being body as an unique material and created a completely novel strategy. It is novel that the inventor first found that the short arm nucleolus tissue region of human D, G group chromosomes is the best target site for human gene therapy and expression (10 sites are present in the short arms of the nucleolus tissue regions of chromosomes 13, 14, 15, 21, 22). A gene vector capable of transferring a gene of interest site-directedly into these target sites is also novel.

Compared with background technologies, the present invention has some striking advantages:

1. Good stability: using the DNA sequence provided by the invention to construct a vector, the human source gene vector can transfer site-directedly a gene of interest into the short arms of D, G group chromosomes of human somatic cells and allow the gene of interest to be inherited stably with chromosomes;
2. Good safety: the target site contains no vital physiological function-related gene, demonstrating that the target site is safe. Meanwhile Fluorescent In Situ Hybridization (FISH) confirmed the vector could insert a gene of interest site-directedly into a safe target site in cells (FIGS. **3**, **4**), excluding insertion mutation at random integration sites and the harm of recombinant wild-type virus. The expression of a gene of interest in target sites is safe. Although the present examples do not provide clinical demonstration of gene expression strategies, the practical examples in example 4 confirm its safety from another angle.
3. Efficient expression: First, the gene vector provided by the present invention comes from targeting sequence at short arms of human D, G group chromosomes so correspondingly there are 10 target sites in human cells and the insertion efficiency is 5-10 times higher at least than any

other vectors. Second, because the targeting sequence comes from short arms of D, G group chromosomes where genes are actively transcribed, the gene of interest is delivered into target sites where it can express highly efficiently.

4. No immunogenicity: the vector comes from a human being, therefore it has no immunogenicity to a human being.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** is mapping by FISH of a 120 kb DNA fragment cloned in PAC.

FIG. **2** is a structure map of a gene vector (the whole length of the gene vector is 11162 bp); the features of which shown are: pGEM-7 (8267-11162): vector replication elements and prokaryotic screening system; TK (1-2840): Eukaryotic cell negative screening gene which utilizes TK promoter and TK poly A signal; Neo (4342-5910): eukaryotic cell positive screening gene which utilizes sv40 promoter and sv40 poly A signal; GLS (2841-4341, 5911-8267): targeting sequence; Cloning site (5910): insert site of the gene of interest;

FIG. **3** is a FISH result showing mapping of an exogenous tPA gene in positive cloned cells, which demonstrates the vector can target a tPA gene site-directedly into short arms of D, G chromosomes;

FIG. **4** is a FISH result showing mapping of an exogenous FIX gene in positive cloned cells, which demonstrate the vector can target a FIX gene site-directedly into short arms of D, G group chromosomes;

FIG. **5** shows a western blot of purified tPA, lanes 1-4 are purified product of tPA, "−" denotes a negative control;

FIG. **6** shows a western blot of FIX positive cells, F3-F23 are six different cell strains, "−" denotes a negative control.

The examples of the invention are only illustrative and should not be considered to be limiting of the present invention.

EXAMPLE ONE

The preparation of the gene targeting sequence provided in the present invention:

1. Obtaining a PAC clone containing a gene targeting sequence

1.1 Construct a BM-specific pUC19 library by microdissection, PCR and microcloning technologies (Deng H—X, Yoshiura K, Dirks R W, et al. Hum Genet 1992, 89:13.)

1.2 Obtaining and identifying BM-specific single copy DNA

(1) The preparation of colony matrix membrane: Draw squares 14×14 on two pieces of nylon membrane and mark them A and B. Place the two membranes on two plates containing solid LB medium, respectively. Pick at random white clones and transfer the clones into squares of two same coordinates, a total of 14×12 clones. Single copy DNA of 100 ng is added to the line 13 as a positive control. No addition of DNA is used as a negative control. The two plates are respectively placed in a incubator at 37° C. for 10-12 hr, then membrane B is kept at 4° C. Membrane A is taken out of the plate, and processed with filter papers immersed in the following solutions: 10% SDS, 5 min, 0.5N NaOH/1.5M NaCl, 3 min, 1.5M NaCl/0.5M Tris.HCl, 3 min, 2×SSC/0.2M Tris.HCl, 10 min. Membrane A is then dried under vacuum at 80° C., 2 hr and stored at 37° C. for use.

(2) The preparation of gDNA probes

Sample 50-70 ng of gDNA and make up with sterile-water to 11 ml, boil at 100° C., 10 min, denature, label using the following reaction system:

2 mM *dNTP(dATP)*	3 ul
primer mixture	2 ul
klenow enzyme	1 ul
α-^{32}*P-dATP*	3 ul

mix, incubate for 30 min at 37° C. in bath, add 8 ul stop mixture, filter through G-50 column to purify the probe, take one-tenth for liquid scintillation counting.

(3) Hybridization: colony dot matrix membrane is placed in 2×SSC and immersed for 10 min. The debris on the surface of membrane is carefully removed. The membrane is pre-hybridized at 65° C. in 5 ml hybridization liquid for 30 min at least. According to the value of liquid scintillation, based on 1.2×10^6 cpm/ml hybridization liquid, sample probe liquid, boil at 100° C., 10 min, denature, add 5 ml fresh hybridization liquid to colony dot matrix membrane and allow to hybridize for over 12 hrs at 65° C. Then wash the membrane under following conditions: 2×SSC/0.1% SDS, 10 min at room temperature, 2×SSC/0.1% SDS, 10 min at 65° C., 0.1×SSC/0.1% SDS, 10 min at 65° C. Autoradiography is performed at −70° C.; a strong or weak hybridization signal is considered to be single copy.

(4) Sequencing, Southern blotting detection: Clones without hybridization signal are picked from the corresponding position of membrane B, expanded, and plasmid DNA is extracted for DNA sequencing. The obtained DNA sequences are compared with the GenBank database; the clones without similarity to other sequence is considered to be single copy. Finally, inserted DNA is isolated by restriction enzyme digestion. The insert is labeled by α-^{32}P-dATP by random primer method and then hybridized with EcoR1 digested gDNA on a nylon membrane; the clone showing one or two bands is considered to be single copy.

1.3 Obtaining and identifying BM and short arms of group D, G chromosomes specific PAC clone

(1) Screen human PAC gDNA library to obtain positive clone

to label The single copy probe P8-7 of 260 bp is labeled using α-^{32}P-dATP by the random primer method and the probe is purified by G-50 column (middle size of particles) and stored at 4° C. Seven pieces of PAC membranes immersed in 2×SSC for 10 min are pre-hybridized for 3 hr at 55° C. Probe DNA is denatured for 10 min at 100° C. and added to 50 ml hybridization solution purchased commercially according to dosage of 4.6×10^5 cpm/ml and hybridized to PAC membrane for 1 hr at 65° C. The membrane is washed as follows: 2×SSC, 10 min one time at ambient temperature, 2×SSC/0.1% SDS, 10 min at 65° C., twice and then placed onto x-ray film.

Autoradiography is performed for 12 hours and the X-ray film is developed and positive clones counted as instruction goes.

(2) pick at random a number of positive clones from five different plates, purchase PAC clones.

1.4 FISH of PAC DNA to metaphase cells of the PAC confirms DNA was from group D,G chromosome, as shown in the FIG. **1**.

Experimental methods referred to above are found in Molecular Cloning. Second Edition (Cold Spring Harbor Laboratory Press, 1989)

2. Isolation of gene targeting sequence DNA

Main materials: β-agarase (Bio-Labs) Not I Agarase

① Digest PAC 169 Plasmid by Not I enzyme;

② Isolate Insert DNA of 120 Kb by PFGE;

Pulse electrophoresis conditions: electrode buffer solution: 0.5×TBE, high strength Analytical Grade Agarase (Bio-Rad, Low Melting point Agarose LMP) 1%, Switch time: 2 seconds→15 seconds→electrophoresis time: 18 hr, voltage: 6V/cm, angle: 120°, temperature: 14° C.

③ after electrophoresis, stained with EB (0.2 ug/ml), 30 min, excise band of 120 kb.

④ the excised gel piece is treated with β-agarase, and the DNA is precipitated in alcohol.

EXAMPLE TWO

The preparation of gene vector provided by the present invention

1. Construction of gene vector and transfer of gene of interest

1.1 Construction of vector

1.1.1 PAC DNA is digested by Nsi I and Stu I (blunt enzyme), 3.8 kb DNA fragment is recovered by general agarose gel electrophoresis and purification by electro-elution;

1.1.2 Digest pGEM-TK vector DNA by Hind III, make it blunt by Klenow enzyme, to generate a blunt end;

1.1.3 The linearized pGEM-TK/Hind III is further digested by Nsi I;

1.1.4 The digested PAC DNA of 3.8 kb and pGEM-TK were ligated at 16° C. for 17 hr;

1.1.5 Ligated product was transformed into JM109 competent bacteria. The transformed bacteria were incubated on a plate containing ampicillin for 18 hr at 37° C.;

1.1.6 Pick individual clones at random, determine positive clones by Nsi I and Nhe I digestion after plasmid extraction. The recombinant plasmid was named pGEM-TK-3.8 Kb.

1.1.7 Obtain Neo gene by digesting pCDN-GPR plasmid with Xba I and Nhe I.

1.1.8 Ligate Xba I and Nhe I digested Neo gene with Nhe I digested pGEM-TK-3.8 Kb to construct pNS2 gene vector;

2.1 Transfer of TPA and FIX genes

2.1.1 Clone TPA and FIX (CDS) into pcDNA3.1 (−), respectively;

2.1.2 Design the primers TPCF and TPCR to amplify TPA and FIX gene and expression elements (CMV promoter and BGH poly A signal), introduce enzyme Avr II restrictive sites into the two ends of primers, the sequences of the primers are:

```
TpcF:    ATgCATCCTAggggAggTCgCTgAgTAgTg
                 AvrII

TpcR:    TgCATgCCTAggTACCCCCTAgAgCCCAg
                 AvrII
```

2.1.3 Digest the amplified TPA or FIX gene and expression components (CMV promoter and BGH poly A signal) by Avr II, and ligate into pNS2 vector digested by NheI enzyme.

The procedures above are found in J. Sambrook et al. "Molecular Cloning". Second Edition. Cold Spring Harbor Laboratory Press. 1989

3. Extraction of gene vector DNA

3.1 Materials

3.1.1 QIAGE Plasmid Maxi Kit

3.1.2 Culture Media: liquid LB

Trypton	5 g
Yeast extract	2.5 g
NaCl	2.5 g
Add ddH2O to	500 ml
Autoclave	

3.1.3 Ampicillin: 100 mg/ml (100×)

3.2 Procedures

1) Pick and inoculate positive clones into 3 ml LB (Amp^+), incubate 1 hr at 37° C.
2) put 100 ul of primary culture above in 100 ml LB (Amp^+), incubate 16 hr at 37° C., 250 rpm
3) harvest bacteria by centrifugation at 6000 g for 15 min at 4° C.
4) add 100 ml buffer P to resuspend bacterial pellet
5) add 10 ml buffer P2, gently invert flask six times to mix up completely, stand for 5 min at ambient temperature
6) add 10 ml pre-cooled buffer P3, gently invert flask six times, place on ice for 20 min
7) centrifuge at 20000 g for 30 min at 4° C.
8) transfer swiftly supernatant to 40 ml centrifugation tube, centrifuge at 20000 g for 15 min at 4° C.
9) 10 ml buffer QBT equilibrates QIGEN tip 500
10) transfer the supernatant to QIGEN tip 500, filter through the column
11) wash the column with 2×30 ml buffer QC
12) elute the column with 15 ml buffer QF, collect the elution liquid
13) add isopropanol (0.7 times volume) 10.7 ml to elution solution, thoroughly mix up
14) centrifuge at 15000 g for 30 min at 4° C.
15) remove the supernatant, add 5 ml, 70% alcohol to DNA precipitate, centrifuge at 15000 g, 4° C. for 10 min
16) remove 70% alcohol, dry the DNA in the air for 10 min, add a certain amount of TE resolve DNA precipitate

EXAMPLE THREE

Introduce gene vector carrying TPA or FIX gene into host cells and express them in vitro

1. Materials

1.1 cell: HT1080

culture medium: high-sugar DMEM+10% FBS (HT1080)EMEM+10% FBS

1.2 Electroporation apparatus: Bio-Rad company

2. Methods:

1) Cells are inoculated in 75 cm^2 canted-neck flask, cultured and grown to 70%-80% confluence.

2) The cells are harvested and washed twice with HeBs buffer solution, and the cell number is counted.

3) Centrifuge at 1500 rpm, 4° C. for 10 min.

4) Resuspend with proper volume of HeBS, dilute the cell density to 10^6-10^7/ml

5) Take 0.4 ml electroporation cuvette, add 0.8 ml cell suspension, 10 ul vector DNA

6) Electroporate the cells at 260v, 550 uFf, lasting 11-13 ms

7) The electroporated cells above are transferred into a 75 cm^2 canted-neck flask, 14 ml culture medium containing ampicillin/streptomycin is added, and the cells are incubated in 5% CO_2, at 37° C., 24-48 hr.

8) Add G418 to culture medium to a final concentration of 300 ug/ml, screen, replace culture medium every 2-3 days and renew G418. HT1080 cells without gene transfer were used as control

9) The control cells died after 7-10 days. The surviving clone cell number within transformed cells is counted, and maintained on a concentration of G418 of 150 ug/ml.

10) Continue to screen transformed cells with GCV of 500 ng/ml

11) After most of the cell clones die after 7-10 days, add maintenance concentration of GCV of 250 ug/ml. When the remaining surviving cells grow up to 70%-80% confluence, detect the expression activity of transferred genes

3. Results

TPA gene and FIX gene are introduced into HT1080 cells, respectively by electroporation using the human source gene vector. Positive clones were obtained after positive and negative screening. Site-directed integration of two genes were confirmed by FISH (FIGS. **3**, **4**). The results of activity determination are detailed in the following tables 1 and 2.

In negative control of HT1080 cells, TPA activity is 0 u/10^6 cells/24 hr, after the transfer, TPA activity is 408 u/10^6 cells/24 hr. Expression efficiency is 407 at day 95 after the transfer; the expression is very stable (Table 1). FIX activity is increased from less than 0.5 ug/ml up to 2.5 ul/ml, the expression content remains 2.6 ug/ml, see Table 2. The expressing products of two genes have been testified by Western Blotting (FIGS. **5**,**6**). The expressed TPA protein has been purified.

TABLE 1

activity detection of positive cells transformed by TPA(ug/10^6 cells/24 hr)			
Days after transformed	T1	days after transformed	T15
33	408	54	88
37	396	58	204
60	411	95	114
68	430		
74	430		
88	441		
90	440.9		
95	407		

(T1, T15 are pTA positive cell strains)

TABLE 2

activity detection of FIX gene (ug/10^6 cells/24 hr)	
Days after transformed	clone F23
60	2.5
72	2.4
100	2.9
109	2.6

EXAMPLE FOUR

Safety case among human beings

Prof. Xia Jiahui has been engaging in human and medical cytogenetics since 1973. He found and identified 732 abnormal karyotypes first reported in the world, which were submitted by 470 clinical cytogeneticists working in 189 laboratories around China. Among these 732 karyotypes, 41 involve the short arms of D, G group chromosomes. No matter which chromosome among chromosomes 1-22 the fragment translocated into short arms of group D, G chromosomes originated, or how the lengths of fragments from the same chromosome are different, the number of gene contained is from one to thousands, but the phenotype of the carrier is normal, which shows that the genes translocated into short arms of D, G group chromosomes can express normally. So it is safe to use short arms of D, G group chromosomes as targeting sites for gene therapy.

1. Karyotype:46,XX,t(1;12;22;15;11;8) (1qter→1p11::8p23→8pter;12pter→12q11::1p11→pter; 22qter→22p11::12q11→12qter; 15pter→22p11→22pter;11pter→11q21::15q15→15qter;8qter→8p23::11q21→11qter)

phenotype: female, 28 years old, normal phenotype carrier

material provider: Wu subing, Cytogenetics laboratory, Department of gynecology and obstetrics, first affiliated hospital of Zhongshan Medical University, Guangzhou

2. Karyotype: 46, XY, t(1;13) (1pter→1q32::13p11→13pter; 13qter→13p11::1q32→1qter).

Phenotype: female, 24 years old, normal phenotype carrier

Material provider: Xiao Chen, Department of biology, Harbin Medical University, Harbin 150086

3. Karyotype: 46, XX, t(2;15) (2pter→cen→15qter; 2qter→cen→15pter)

phenotype: female, 26 years old, normal phenotype carrier

material provider: Guo Yuping, et al. Cytogenetics Department, Jiangxi provincial gynecology and obstetrics hospital, Nanchang 330006, Jiangxi province

4. Karyotype: 46,XY,t(2;21) (2pter→cen→21pter; 2qter→cen→21qter)

phenotype: male, 32 years old, normal phenotype carrier

material provider: Kang Guoqing, et al. Department of genetics, the second affiliated hospital of Shangxi Medical College, Taiyuan 030001

5. Karyotype: 46,XY,t(3;21) (2qter→cen→22pter; 3qter→cen→22qter)

phenotype: male, 26 years old, normal phenotype carrier

material provider: Gao Yun. Department of toxicology, Bingzhou municipal Medical College, Bingzhou 256603, Shangdong province

6. Karyotype: 46,XY,t(3;22) (3pter→cen→22pter; 3qter→cen→22qter)

phenotype: male, 29 years old, normal phenotype carrier

material provider: Shi Huajin. Department of genetics, Jingzhou Women and Baby hospital, Jingzhou 121000, Liaoning province

7. Karyotype: 46,XX,t(4; 15)(4qter→4p13:: 15p13→15pter; 15qter→15p13::4p13→4pqter)

phenotype: female, 28 years old, normal phenotype carrier

material provider: Zhou Ling, et al. Laboratory of genetics, the Wuhan Children hospital, Wuhan 430016, Hupei province

8. Karyotype: 46,XY,t(4;21)(4qter→4p15:: 21p11→21pter;21qter→21p11::4p15→4pqter)

phenotype: female, 25 years old, normal phenotype carrier

material provider: Xu Jinfang, et al. Laboratory of genetics, the sixth people's hospital of Shanghai, Shanghai 200000

9. Karyotype: 46,XY,t(4;14)(4qter→4q31:: 14p11→14pter;14qter→14p11::4q31→4qter)

phenotype: male, normal phenotype carrier

material provider: Zhou Mingjun, et al. Xuchang Municipal Central Hospital, Xuchang 161000, Henan province

10. Karyotype: 46,XY,t(4;14)(4qter→4q35:: 14p11→14pter;14qter→14p11::4q35→4qter)

phenotype: male, 27 years old, normal phenotype carrier

material provider: Zhang Xiuquan, et al. Hushan Municipal Women and Nursling Hospital, Hushan 528000, Guangdong province

11. Karyotype: 46,XX, t(6; 22)(5qter→5q13:: 22p11→22pter; 22qter→22p11::5q13→5qter)

phenotype: female, 32 years old, normal phenotype carrier

material provider: Zhao Jianping, Anyang Municipal Women and Nursling Hospital, Anyang 455000, Henan province

12. Karyotype: 46,XY, t(6;22) (6pter→cen6→22qter; 6qter→cen22→22pter)

phenotype: male, 25 years old, normal phenotype carrier

material provider: Zhu Xinxia, et al. Laboratory of cytogenetics, Department of Gynecology and Obstetrics, Number 88 Hospital, Taian 271000, Shangdong province

13. Karyotype: 46,XY, t(6; 22)(6qter→6p21:: 22p11.2→22pter; 22qter→22p11.2::6q21→6pter)

phenotype: male, 33 years old, normal phenotype carrier

material provider: Yang Qinglan, Department of Gynecology and Obstetrics, affiliated hospital of Bingzhou Medical College, Bingzhou 256603, Shangdong province

14. Karyotype: 45,XX,t(7;21) (7qter→7p22:: 21p12→21qter)

phenotype: female, 23 years old, normal phenotype carrier

material provider: Sun Qingji, et al. Laboratory of genetics, the Wuhan Children hospital, Wuhan 430016, Hubei province

15. Karyotype: 46,XY/46XX,t(7;14)(7pter→7q11:: 14p11→14pter;14qter→14p11::7q11→7qter)

phenotype: male, 28 years old, normal phenotype carrier

material provider: Li Luyun, Xia Jiahui, et al. State Key Laboratory of Medical genetics (Hunan Medical University), Changsha 410078, Hunan province

16. Karyotype: 46,XY,t(8;14)(8pter→8p21:: 14p12→14pter;14qter→14p12::8p13→8pter)

phenotype: male, 27 years old, normal phenotype carrier

material provider: Shi Huajin, et al. Department of genetics, Jingzhou Women and Baby hospital, Jingzhou 121000, Liaoning province

17. Karyotype: 46,XY,t(9;14)(9pter→cen→14pter; 9qter→cen→14qter)

phenotype: male, 28 years old, normal phenotype carrier

material provider: Cheng Qiuyun, et al. Department of reproduction medicine, first affiliated hospital of Hengyang medical college, Hengyang 421001, Hunan province

18. Karyotype: 46,XY,t(9;22) (9pter→9p13:: 22p12→22pter;22qter→22p12::9p13→9pter)mat

phenotype: female, 31 years old, her mother, a young sister of her, a young brother of her and her son have the same phenotype as her, that is normal phenotype carrier

material provider: Li Luyun, Xia Jiahui, et al. State Key Laboratory of Medical genetics (Hunan Medical University), Changsha 410078, Hunan province

19. Karyotype:46,XX,t(9;14) (9pter→9q12:: 14p12→14pter; 14qter→14p12::9q12→9qter).
Phenotype: female, 32 years old, normal phenotype carrier

Material provider: Sun Yanyang, et al, Department of biology, Harbin Medical University, Harbin 150086

20. Karyotype:46,XX,t(9;15) (9pter→9q21:: 15p12→15pter; 15qter→15p12::9q21→9qter)mat.
Phenotype: female, 36 years old, normal phenotype carrier

material provider: Zhu Guizhen, et al. Laboratory of cytogenetics, Department of Gynecology and Obstetrics, Number 88 Hospital, Taian 271000, Shangdong province

21. Karyotype:46,XX,t(10;13) (10pter→10q24:: 13p11→13pter; 13qter→13p11::10q24→10qter)
Phenotype: female, 28 years old, normal phenotype carrier

material provider: Yan Dunqing. Department of Gynecology and Obstetrics, affiliated hospital of Qingdao Medical College, Qingdao 266003, Shangdong province

22. Karyotype: 46,XX,t(10;13) (10pter→10q24:: 13p12→13pter; 13qter→13p12::10q24→10qter)
Phenotype: female, 29 years old, normal phenotype carrier

material provider: Zhang Yinru, et al. Department of neurology First affiliated hospital of Zhongshan Medical University, Guangzhou 510080, Guangdong province

23. Karyotype: 46,XX,t(11;14) (11pter→cen→14pter:: 11qter→cen→>14qter)

material provider: Wang Zhiyong, Department of genetics, Zhacheng County people's hospital, Zhacheng County 476200, Henan province

24. Karyotype: 46,XX,t(11;21) (11pter→11p11:: 21p11→21pter; 21qter→21p11::11p11→11pter)

Phenotype: female, 26 years old, normal phenotype carrier

material provider: Zheng Jun, et al. Department of genetics, Shanxi provincial women and nursling hospital, Xian 710003 Shanxi province

25. Karyotype: 46,XX,t(11;15) (11pter→11q13:: 15p12→15pter; 15qter→15p12::11q13→11qter)

Phenotype: male, 23 years old, normal phenotype carrier

material provider: Yang Ruifang, et al. Medical center of Obstetrics, affiliated hospital of Shandong Medical University, Jinan 250012, Shandong province

26. Karyotype:46,XX,t(12;14) (12pter→cen→14pter:: 12qter→cen→14qter)

Phenotype: female, 28 years old, normal phenotype carrier

material provider: Han Weitian, et al. Department of eugenics, Liaoning provincial institute of family planing, Shenyang 110031, Liaoning province

27. Karyotype:46,XX,t(13;16) (13qter→13p11:: 16p11.2→16pter;16qter→16p11.2::13p11→13pter

Phenotype: female, 27 years old, normal phenotype carrier

material provider: An Songlan. Department of genetics, Dalian municipal gynecology and obstetrics, Dalian 110078, Liaoning province

28. Karyotype: 46,XY/46,XX,t(13;13) (13qter→13p12:: 13p12→13qter)

Phenotype: male, 39 years old, normal phenotype carrier

material provider: Li Luyun, Xia Jiahui, et al. State Key Laboratory of Medical genetics (Hunan Medical University), Changsha 410078, Hunan province

29. Karyotype:46,XY,t(14;18) (14pter→cen→18pter; 14qter→cen→18qter)

Phenotype: male, 30 years old, normal phenotype carrier

material provider: Wang Sugui, et al. Beijing Institute of family planing technology guidance, Beijing 100006

30. Karyotype:46,XX,t(14;15) (14pter→14q13:: 15p13→15pter;15qter→15p13::14q13→14qter)

Phenotype: female, 28 years old, normal phenotype carrier

material provider: Li Luyun, Xia Jiahui, et al. State Key Laboratory of Medical genetics (Hunan Medical University), Changsha 410078, Hunan province

31. Karyotype:46,XX,t(15qter→cen→22qter)

Phenotype: female, 27 years old, normal phenotype carrier

material provider: Li Luyun, Xia Jiahui, et al. State Key Laboratory of Medical genetics (Hunan Medical University), Changsha 410078, Hunan province

32. Karyotype:46,XY,t(15;18) (15pter→cen→18pter; 15qter→cen→18qter)

Phenotype: male, 30 years old, normal phenotype carrier

material provider: Ren Guoqing, et al. Beijing Institute of family planing technology guidance, Beijing 100006

33. Karyotype:46,XX,t(15;20) (15pter→cen→2pter; 15qter→cen→2qter)

Phenotype: female, 26 years old, normal phenotype carrier

material provider: Wang Xin, et al. Laboratory of genetics, department of obstetrics, the second affiliated hospital, Hunan Medical University, Changsha 410011, Hunan province

34. Karyotype:46,XX,t(15;22) (15pter→15q11:: 22p13→22pter;22qter→22p13::15q11→15qter)

Phenotype: female, 27 years old, normal phenotype carrier

material provider: Hu Shengdi, Department of genetics, Hainan provincial people's hospital, Haikou 570011, Hainan province

35. Karyotype:46,XX,t(15;22) (15pter→15q22:: 22p11→22pter;22qter→22p11::15q22→qter)

Phenotype: female, 29 years old, normal phenotype carrier

material provider: Li Murou, Department of genetics, Xinjiang Medical College, Urumchi 830054

36. Karyotype:46,XY,t(16;21) (16pter→16q11:: 21p11→21pter;21qter→22p11::16q12→16qter)

Phenotype: male, 29 years old, normal phenotype carrier

material provider: Zhang Huifang, et al, Institute of family planing technology of Guangdong, Guangzhou 510080, Guangdong province

37. Karyotype:46,XX,t(18;21) (18pter→cen→21pter; 18qter→cen→21qter)

Phenotype: female, normal phenotype carrier

material provider: Shi Huajin, et al. Laboratory of genetics, Jingzhou women and nursling hospital, Jingzhou 121000, Liaoning province

38. Karyotype:46,XX, t(18;21) (18pter→18q11:: 21p12→21pter;21qter→22p12::18q11→18qter)

Phenotype: female, 26 years old, normal phenotype carrier

material provider: Li Xiulin, et al, laboratory of genetics, department of pediatrics, first affiliated hospital of Chinese medical university, Shenyang 110011, Liaoning province

39. Karyotype:45,X,dic(Y;13)(Ypter→Yp1200:: 13p11→cen→13qter)

Phenotype: male, 4 years old, normal phenotype carrier

material provider: Xia Jiahui, et al. State Key Laboratory of Medical genetics (Hunan Medical University), Changsha 410078, Hunan province

40. Karyotype:46,XY,t(Y;15)(15qter→15p12:: Yq12→Ypter) pat.

Phenotype: male, 4 years old, normal phenotype carrier

material provider: Xia Jiahui, et al. State Key Laboratory of Medical genetics (Hunan Medical University), Changsha 410078, Hunan province

Abnormal chromosome carriers described above showed no abnormal syndrome, which shows that not only can nucleolus tissue receive foreign genes but also it allows foreign genes to express normally.

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The invention claimed is:

1. A vector consisting of:

i) targeting nucleotide sequences consisting of:

a sequence consisting of nucleotide positions 75590 through 79448 of SEQ ID NO:1; and

a sequence consisting of nucleotide positions 77091 through 79448 of SEQ ID NO:1, wherein the targeting sequences provide for integration into the short arm of a human Group D or Group G chromosome;

ii) a marker gene providing for a positive selection for eukaryotic or human host cells that harbor the vector;

iii) a gene providing for negative selection for eukaryotic or human host cells that harbor the vector;

iv) a restriction enzyme site in the targeting nucleotide sequence, wherein the restriction enzyme site is unique in the vector; and

v) a polynucleotide sequence that provides for replication of the vector in a prokaryotic host cell.

2. The vector of claim **1** that further comprises a desired polynucleotide inserted into the unique restriction site.

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